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Research paper

Physico-chemical characterisation of cationic DOTAP liposomes as drug delivery system for a hydrophilic decapeptide before and after freeze-drying

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ABSTRACT

In the present study, positively charged 1,2-dioleoyloxy-3-trimethylammoniumpropane (DOTAP) liposomes as a delivery system for a hydrophilic decapeptide were developed. The main objective was the preparation of a stable, highly loaded, lyophilised formulation to yield the basis for an acceptable shelf life. The influences of addition of cholesterol, pH value, amounts of lipid and peptide, type and amount of sugar-based cryoprotective agent (trehalose and sucrose), and time point for cryoprotector addition as well as the freeze-drying process parameters were investigated. The collapse temperatures of the liposome dispersions in the presence of the disaccharides trehalose and sucrose were determined using a freeze-drying microscope (Lyostat 2). The liposome morphology before freeze-drying was determined by transmission electron microscopy (TEM). The evidence of intact liposomes after freeze-drying was shown by scanning electron microscope (SEM) imaging. In summary, this study demonstrated the successful development of DOTAP liposomes including their lyophilisation as a drug delivery system for small hydrophilic peptides.

1. Introduction

Peptide leakage and lyophilisation feasibility are the main challenges for long-term shelf life of peptide liposomal formulations. Although in the past few decades the interest in liposomes as drug delivery system for a wide variety of molecules in different application areas has grown exponentially, only few formulations were successfully approved by the regulatory agencies for parenteral applications, and the number of clinical studies actually is sparse in relation to the amount and invested time of research until today. Marketed formulations include the antifungal freeze-dried liposome formulation AmbiSome® and some formulations with doxorubicin as the active pharmaceutical ingredient (DOXIL® (USA) = Caelyx® (Europe), Myocet®). In the vaccine sector, only few liposomal formulations (Epaxal® or Inflexal V®) were successfully licensed and manufactured until now. Especially, the difficulties in the encapsulation of hydrophilic compounds into the inner aqueous core represent a major challenge and often require process amendments such as ultrasonic treatment or the addition of a detergent for surmounting the lipophilic bilayer membranes. In addition, hydrophilic compounds may diffuse out of the liposomes during storage over time. Hence, the most successful liposomal i.v. products are prepared with lipophilic compounds as they can easily interact and adhere to the liposome shell in comparison to hydrophilic ones.

Furthermore, the potential application of liposomes for therapy is limited by their physical and chemical instabilities in aqueous dispersions [1]. Because of the phospholipids, the fatty acid chains tend to oxidise or hydrolyse increasingly with unsaturated components leading to a rise in bilayer permeability [2]. Beside this chemical instability of the liposomes, physical instability like fusion, antigen leakage or precipitation of ingredients may occur during storage [1]. To overcome these chemical and physical instabilities, the approach to freeze-dry liposome formulations is an important improvement.

In the present study, the positively charged DOTAP lipid was chosen for the development of a liposome vaccine formulation. This cationic lipid exhibits a high affinity to negatively charged cell membrane compounds (especially angiogenic endothelial cells) and therefore enhances the internalisation and presentation of the active ingredient by molecular, passive or electrostatic interactions resulting in an efficient induction of cell-meditated processes (e.g. EndoTAG®) [3–5]. Thus, the drug could effectively operate on its targeted location, e.g., dendritic cells or antigen-presenting cells. Moreover, several scientists demonstrated the potent immunological adjuvant effect of DOTAP liposomes on dendritic cells and by predictions from in vitro/in vivo studies [6–8].

The model peptide used in this study was a linear and hydrophilic decapeptide and was incorporated into DOTAP liposomes. Since this

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peptide was sensitive to hydrolytical degradation, a freeze-drying process was developed and evaluated in order to obtain a long-term stable system that still contained the encapsulated peptide after reconstitution in the inner core of the liposomes. The influence, the time point and the amount of the sugar-based cryoprotective agent (trehalose or sucrose) were explored. Furthermore, the freeze-drying process parameters like primary drying temperature, freezing rate and secondary drying were varied, and their influence on the resulting products was investigated.

2. Materials and methods

2.1. Materials

For all experiments, the lipid 1,2-dioleoyloxy-3-trimethylammoniumpropane (DOTAP) chloride (Mw: 698.54 g/mol) was used as obtained from Merck & Cie (Schaffhausen, Switzerland). Methanol, acetonitrile (both LiChrosolv®, gradient grade), chloroform (SeccoSolv®, max 0.003% H_2O), ammonia acetate (Pro analysi), ammonia solution 25% (EMSURE®), trehalose anhydrous and sucrose (both for biochemistry purpose) were from Merck KGaA (Darmstadt, Germany). Cholesterol (Mw: 386.67 g/mol) was purchased from Avanti Polar Lipids (Alabaster, USA). Purified water of MilliQ quality (MILLIPORE® Q-POD™) was used for all experiments. The peptide (IATFKNWPFL-OH, Mw: 1236.5 g/mol, purity determined by HPLC 99.2%) was purchased from Peptisyntha, Inc. (Torrance CA, USA). For purifying the liposome dispersions by dialysis, Spectra/Por® dialysis membrane (MWCO 6-8000, Spectrum Laboratories Inc., USA) was used.

Table 1
Composition of the DOTAP liposome formulations.

Preparation: DOTAP	D1	D2a	D2b	D3a	D3b
Panel a					
DOTAP amount (μmol/ml)	28.3	21.2	14.2	28.3	28.3
Initial peptide concentration (mg/ml)	0.25	0.25	0.25	0.5	0.75
pH value	9	9	9	9	9

Preparation: DOTAP/ cholesterol (1 mol:1 mol)	DC1a	DC1b	DC2a	DC2b	DC3a	DC3b
Panel b						
Preparation: DOTAP amount (μmol/ml)	14.2	14.2	10.6	7.1	14.2	14.2
Initial peptide concentration (mg/ml)	0.25	0.25	0.25	0.25	0.5	0.75
pH value	5	9	9	9	9	9

Preparation: DOTAP/ cholesterol (1 mol:1 mol)	DC1bT3	DC1bT5	DC1bS3	DC1bS5	DC1bS7
Panel c					
DOTAP amount (μmol/ml)	14.2	14.2	14.2	14.2	14.2
Initial peptide concentration (mg/ml)	0.25	0.25	0.25	0.25	0.25
pH value	9	9	9	9	9
Cryoprotective agent	Trehalose	Trehalose	Sucrose	Sucrose	Sucrose
Cryoprotective agent amount (%) (w/v)	3	5	3	5	7

2.2. Methods

2.2.1. Preparation of peptide loaded DOTAP liposomes

The hydrophilic decapeptide was incorporated into the drug delivery system of DOTAP liposomes using the lipid film hydration method [9] with minor modifications. Briefly, the required lipid amounts (Table 1) with or without cholesterol were dissolved in a defined volume of chloroform and filled into a 100 ml round-bottom flask. The employed volume for chloroform always corresponded to half of the volume for the aqueous phase. The organic solvent was removed under vacuum (1–10 mbar) by a rotary evaporator (Rotavapor R-134, Büchi, Essen, Germany) at 40 °C (water bath) for 1 h. A thin film was formed on the inner side of the round-bottom glass vessel. Afterwards, the film was stored at least for 12 h at 2–8 °C. The next day, the film was left to room temperature equilibration and hydrated in a defined volume of aqueous peptide solution (0.25, 0.5, 0.75 mg/ml, set to pH 9 with ammonia solution 25%) containing a cryoprotective agent (sucrose or trehalose; 3%, 5%, 7% (w/v)) if required. The mixture was shaken by hand until all dried lipid film was removed from the glass vessel and was dissolved. The dispersion became turbid indicating that liposomes were formed. To obtain a homogenous liposome suspension, the mixture was then dispersed with an Ultraturrax (IKA®T10 basic Ultra-turrax®) for 10 min (grade 6, motor speed about 30,000 min^{-1}).

To remove the free peptide, i.e., peptide that was not encapsulated in the inner core of the liposomes, 3 ml dispersion was filled into a dialysis membrane (on both ends clip-closed) and dialysed for 24 h with 5 l of aqueous media of the same composition as the aqueous liposome content without peptide to allow the free peptide to diffuse out. The obtained liposome dispersion then was analytically characterised or freeze-dried.

2.2.2. Physico-chemical characterisation of DOTAP liposomes

The particle size, size distribution (polydispersity index, PDI) and zeta-potential of the obtained liposomes were determined before and after freeze-drying ($T = 25$ °C, 173° detection angle of laser) by photon correlation spectroscopy (PCS) using a Malvern Zetasizer Nanoseries-ZS (Malvern Instruments, Worcestershire, UK). Briefly, 2 μl of the liposome suspension was diluted in 998 μl deionised water (filtered before usage trough a 0.22 μm polystyrene filter) and filled in a 1.5 ml semi-micro disposable cuvette (for zeta-potential measurements folded capillary cells were used). The mean hydrodynamic diameter of the particles and the *z*-average (average of two measurements, each involving 10 runs) were recorded.

The liposomes morphology before freeze-drying was analysed by transmission electron microscopy (TEM, CM20, limitation 2.3 A, FEI®, Netherlands) to clearly show the liposome morphology including the lipid bilayers. TEM cannot be used for solid preparations, and therefore, after lyophilisation a scanning electron microscope (SEM Supra 35, Carl Zeiss NTS GmbH, Germany) was investigated to demonstrate the integrity of the liposomes character after freeze-drying. To obtain a very thin suspension for TEM measurement, the liposome dispersion was diluted 1:5 (v/v) with milliQ-water and contrasted 1:1 (v/v) with 4% of a phosphotungstic acid (pH 7.2). This dispersion was applied to a copper grid and images were taken after drying. For SEM pictures, the freeze-dried liposome cake was placed on a conductive sample appliance made of aluminium. The adhered sample amount was sputtered with platinum and the prepared freeze-dried cake was analysed by SEM.

To monitor the pH value of the liposomes dispersion, a 780 pH metre with a micro glass electrode (pH 1–11, Metrohm AG; Zofingen, Switzerland) was used. The existing pH value of the formulation was determined potentiometrically after calibration with known standards.

The osmolarity of the formulations was determined with an Osmomat type 030/D osmometer (Gonotec GmbH, Berlin, Germany). The sample volume was 50 μl.

The dynamic viscosity of the liposome formulation was measured using a rotational cone and plate viscometer RS1 (Thermo Haake GmbH, Germany). The shear stress at increasing shear rates, i.e., 500–1000 (1/s) (slot 0.027 mm, sensor size C60/0.5°Ti), was measured in 600 μl of the samples.

The residual moisture content in the freeze-dried liposome dispersions was analysed by indirect colorimetric Karl-Fischer-titration realised by a 756 KF coulometer with a 744 oven sample processor (Metrohm AG, Switzerland). 70 mg of the lyophilised samples was determined in triplicate at 140 °C or 100 °C with a gas stream (flow rate 69 ml/min) of dry 0.3 nm nitrogen/air. The average values reported are percentage of residual water content in the samples after freeze-drying.

2.2.3. Encapsulation efficiency of the hydrophilic decapeptide

The encapsulation efficiency (EE) before and after lyophilisation and rehydration of the freeze-dried cakes was measured by the amount of peptide recovered in dissolved liposomes and was quantified by HPLC (LaChrome Elite, Merck Hitachi High-Technologies Corporation, Japan). Briefly, 1 ml acetonitrile was added to 500 μl purified liposome dispersion to dissolve the DOTAP lipid and precipitate cholesterol. The samples were vortexed and placed for 10 s in an ultrasonic bath (Ultrasonic cleaner USC2100TH, VWR International GmbH, Germany). To quantify the released peptide from the disintegrated liposomes, the samples were centrifuged (14,000 rpm, 15 min, $T = 21$ °C, Eppendorf 5804R, VWR International GmbH, Germany) to spin down the precipitated lipids, and the released peptide was quantified in the supernatant.

The mobile phases for HPLC analysis consisted of A) acetonitrile/ammonia acetate 5 mM [95/5] and B) acetonitrile/ammonia acetate 5 mM [5/95]. A ZIC-HILIC (250 × 4.6 mm, 5 μm, 200 A Merck KGaA) column was used. The flow rate was 1.0 ml/min; the detection wavelength 205 nm and the sample injection volume 20 μl.

Results were calculated from linear regression of a peptide concentration range between 5 μg/ml and 0.5 mg/ml. All peptide quantification tests were run in triplicate and the mean ± SD were reported.

The encapsulation efficiency (EE) was calculated using the followed equation Eq. (1):

$$\mathrm{EE}\ (\%) = \frac{\text{effective peptide concentration (mg/ml) in liposomes}}{\text{initial peptide concentration (mg/ml) in liposomes}} \times 100 \quad (1)$$

2.2.4. Determination of the collapse temperature of formulated liposome dispersions

To determine the collapse temperature (T_C) of the DOTAP liposome dispersions in presence of cyroprotective agents, a freeze-drying microscope (Lyostat 2, Biopharma Technology Ltd., Winchester, United Kingdom) was used. The microscope consisted of an imaging station, a freeze-drying element (freeze-drying cryo stage, FDCS196) with a liquid nitrogen pump cooling system (LNP95, Linkam Scientific Instrument Ltd., Surrey, United Kindom), a temperature-controlled block and a light source (TH4-200 halogen lamp power, Olympus, Hamburg, Germany) in the bottom. The samples were observed by an objective lens with a magnification of 10×, and their collapse temperature was determined by analysing the captured images (1 s intervals) with the LinkSys32 software (temperature control and video capture software). Briefly, 2 μl of the sample solution was placed between a quartz – (16 mm diameter, on the bottom) and a glass – (13 mm diameter, on top) cover slip in the centre of the freeze-drying chamber. Additionally, a custom-made metal spacer (70 μm thick) was added between the slips to guarantee constant sample thickness. To enhance thermal contact and temperature lubrication, silicon oil was dropped between the temperature block and the quartz cover slide.

To specify the collapse temperatures, the valve was closed for vacuum and the samples were frozen at a ramp rate of 20 °C/min to −40 °C, kept at this temperature for 2 min and vacuum was applied down to <0.02 mbar. Before heating the sample at a rate of 1 °C/min up to 20 °C, an optimal area was adjusted for observation. After starting the drying process, sublimation occurred and the sublimation front got visible. The first structural defects or sample scarification (observable holes or fissures) within the sublimation front indicated the point of collapse. Finally, the collapse temperature was determined as the temperature directly before the structural modification.

2.2.5. Freeze-drying of peptide loaded DOTAP liposomes

Various experiments were carried out to determine the best parameters to prepare a stable liposome formulation including (1) the freeze-drying process parameters, (2) different kinds of sugar-based cryoprotective agents in different amounts (sucrose and trehalose; 3%, 5% and 7% (w/v)) and (3) the time point in adding the cryoprotective agent (after or during preparation) to the formulation.

As indicators of an effective lyophilisation, the liposome size, PDI and entrapped peptide after lyophilisation and additional dialysis were selected.

1 ml of the freshly prepared liposome dispersion was filled into 2-ml glass vials (Fiolax, HGB 1/ISO 719, MGlas AG, Münnerstadt, Germany). In the cases where the cryoprotective agent was not incorporated during the liposome preparation, the required amount of sugar (3%, 5% and 7% (w/v)) was weighed directly in the glass vials before adding the liposome dispersion. The vials were closed with a rubber stopper and shortly vortexed. After 30 min of resting the vials were placed in the drying chamber of the freeze-drier (Com 6011; Hof Sonderanlagenbau GmbH, Lohra, Germany). The freeze-drying protocols for the realised lyophilisation processes are given in Table 2.

In addition, in process 1 and 2, an annealing step was included. For this purpose, the temperature was increased up to −15 °C for 2 h while freezing followed by cooling down to its original adjusted temperature. The step was included to allow the ice crystals to grow in order to achieve a more porous cake. Due to the higher freezing rates of process 1 and 2, the cakes of these runs theoretically were stiffer which results in a longer primary drying cycle. To avoid the longer primary drying step, the annealing step was involved. For process 3, this procedure was omitted and the freezing rate was lowered.

After the freeze-drying process images of the frozen cakes were captured and the samples were stored at 2–8 °C. For further analysis (size determination, zeta-potential measurement, osmolarity, viscosity and pH value), the lyophilisates were reconstituted in 1 ml of milliQ-water.

To determine the peptide amount that was entrapped within the liposomes after lyophilisation, the reconstituted dispersion was filled into a dialysis membrane and dialysed for 3 h (dialysis

Table 2
Parameters of the different freeze-drying cycles for the liposome lyophilisation process.

Process no.	1	2	3
Process step			
Freezing			
Plate temperature (°C)	−40	−50	−50
Rate (°C/min)	1.5	3.7	0.5
Duration (h)	2 Annealing step involved	3 Annealing step involved	2
Primary drying			
Plate temperature (°C)	−23	−38	−38
Chamber pressure (mbar)	0.04	0.04	0.08
Duration (h)	12	48	48
Secondary drying			
Plate temperature (°C)	25	25	35
Chamber pressure (mbar)	0.04	0.04	0.08
Duration (h)	4	12	6

time validated, data not shown) with 5 l of the aqueous media of the same composition as the aqueous liposome content without peptide to purify the dispersion from leaked peptide. The purified liposomes were then analysed by HPLC for the remaining peptide.

2.2.6. Statistical analysis

All formulations were prepared and reported in triplicate. Results are expressed as mean ± SD (standard deviation). All data were plotted in Microsoft Excel and statistically analysed using the *t*-test with a significance level of $p < 0.05$.

3. Results and discussion

3.1. Liposome preparation and characterisation

3.1.1. Variation in pH

At first, the effect of the pH of the aqueous peptide phase on the liposomes was investigated. For this purpose, liposomes with cholesterol (DC) were prepared at pH 5 (DC1a) and 9 (DC1b) and compared with liposomes prepared without cholesterol (D1) at a pH of 9. In Table 3 (Panel 1), the liposome size and the PDI for the formulations DC1a, DC1b and D1 (Table 1) are shown. For the formulations prepared with cholesterol (DC), the pH ranging between pH 5 (DC1a) up to pH 9 (DC1b) did not show any significant effect on the liposome size (both around 480 nm, $p > 0.05$). However, a significant decrease in liposome homogeneity (PDI = 0.20 ± 0.01, $p < 0.05$) at pH 9 was observed. If only the DOTAP lipid was used for liposome preparation (D1), the size significantly decreased (303 ± 11 nm, $p < 0.05$) combined with a significant increase in PDI (0.31 ± 0.01, $p < 0.05$).

Table 3
Size, PDI and encapsulation efficiency results of the liposomal formulations (mean ± SD; $n = 3$).

Liposomal formulation	Size (nm)	PDI	Encapsulation efficiency (%)
DC1a	481 ± 13	0.14 ± 0.03	14.4 ± 0.3
DC1b	482 ± 13	0.20 ± 0.01	20.5 ± 1.5
D1	303 ± 11	0.31 ± 0.01	21.2 ± 0.7
DC2a	499 ± 28	0.20 ± 0.01	21.5 ± 4.1
DC2b	291 ± 19	0.34 ± 0.03	15.6 ± 2.5
D2a	314 ± 28	0.36 ± 0.09	23.3 ± 1.7
D2b	261 ± 14	0.36 ± 0.06	16.3 ± 1.7
DC3a	342 ± 5	0.25 ± 0.01	18.8 ± 1.7
DC3b	286 ± 31	0.29 ± 0.01	18.8 ± 2.3
D3a	230 ± 11	0.37 ± 0.05	17.7 ± 0.5
D3b	190 ± 15	0.39 ± 0.09	16.7 ± 0.8

In the presence of cholesterol, the membranes of the liposomes became more homogenous and stable because of its good membrane stabilisation effect. Accordingly, the volume of the inner aqueous liposome core increased, and membrane fusion for the lipids was prevented, and as a result, more homogenous liposomes with bigger sizes were obtained in presence of cholesterol.

Regarding the effect of different pH values on the encapsulation efficiency (EE), higher values ($p < 0.05$) were observed with pH 9 (DC1b and D1) independent of cholesterol addition (Table 3, first panel). A 1.4-fold increase in EE with pH 9 versus pH 5 was achieved by addition of cholesterol (DC1b versus DC1a).

This significant influence of the pH on EE was due to the peptide's isoelectric point of *pI* = 7. At pH 9, the peptide was negatively charged and could easily interact with the positively charged DOTAP lipid. Consequently, the EE of the hydrophilic decapeptide also was influenced by the cationic DOTAP lipid as demonstrated in Table 3 (DC1b versus D1). Equal values were obtained for DOTAP:cholesterol 1:1 (mol/mol) (DC1b) liposomes compared with D1 with the double amount (mol) of DOTAP lipid without cholesterol ($p > 0.05$). Even with higher amounts of DOTAP (D1, 28.3 μmol/ml), it was not possible to increase the EE, and consequently, a saturation of peptide encapsulation was obtained at 14.2 μmol/ml DOTAP (DC1b).

3.1.2. Variation of lipid amount

The effect of variation of the lipid amount was analysed in the next experiment. In Table 3 (Panel 2), the resulting values for particle size and PDI are shown. The initial investigated formulations DC1b and D1 (Table 3, Panel 1) both had a total lipid amount of 28.3 μmol/ml, whereas the DC formulations consisted of a DOTAP:cholesterol ratio of 1:1 (mol/mol). For the formulations DC2a and D2a (Table 3, Panel 2) the total lipid amount was adjusted to 0.75-fold of the initial lipid amount; for the formulations DC2b and D2b, 0.5-fold lipid reduction was chosen.

For both formulations DC2a and D2a with a 0.75-fold lipid content, the liposome sizes were not significantly affected compared with their formulations with the initial lipid amount DC1b and D1 ($p > 0.05$). Not until a 0.5-fold lipid reduction was employed (DC2b and D2b), a significant decrease in liposome size ($p < 0.05$) with increasing PDI values for both formulations DC ($p < 0.05$) and D ($p > 0.05$) was observed. This decrease in size was more evident for the formulation with cholesterol (about 200 nm of decrease). Generally, the formulations manufactured without cholesterol (D) led to smaller liposomes in size with increasing PDI values independently of the adopted lipid amount (Table 3, Panel 2).

In contrast, Table 3 demonstrates a decrease in total lipid amount led to decreasing EE values for the DC and D formulations at constant peptide concentrations. The EE was not significantly ($p > 0.05$) higher with the formulations with the 0.75-fold initial lipid amount independently of cholesterol presence (DC2a and D2a). The results between DC and D formulations were comparable and confirmed the assumption that mainly the DOTAP lipid was responsible for either peptide binding or its entrapment. Due to the fact that the zetapotenial of empty liposomes (55 ± 1) was equal to those loaded with peptide (56 ± 5), it can be concluded that the peptide was entrapped within the liposomes.

In summary, liposomes became bigger and more homogenous by increasing lipid amount. If the total lipid amount was lower, then 75% of the initial lipid amount, the liposomes fused and agglomerated and the whole dispersion became inhomogeneous with an increasing PDI. Only the formulations DC1b (total lipid

amount 28.3 μmol/ml) and DC2a (0.75-fold initial lipid amount) were stable and homogenous in size and PDI.

3.1.3. Variation of peptide amount

In the following experiments, the initial peptide concentration (DC1b and D1) was increased at constant lipid amount and pH. Table 3 (Panel 3) shows that the peptide concentration had an influence on liposome size and PDI. An increase in initial (DC1b and D1; Table 3, Panel 1) peptide concentration led to a significant decrease in size accompanied by an increase in PDI (DC 3a and DC 3b, ($p < 0.05$ for size and PDI); D3a and D3b ($p < 0.05$ for size and $p > 0.05$ for PDI)), i.e., at increasing peptide amount, the liposomes became more inhomogeneous in presence of cholesterol.

This phenomenon can be explained by a higher available peptide charge resulting in stronger peptide-DOTAP electrostatic bonds and formation of smaller liposome sizes. All PDIs in Table 3 (Panel 3) of D formulations were higher compared with the ones of the DC formulations. This indicated more inhomogeneous liposomes with high diversity of existing sizes regarding to DC formulations.

In Table 3 (Panel 3), the effect of increasing peptide amounts on the EE at a constant lipid amount and pH value is depicted. With increasing peptide amounts, the EE slightly decreased ($p > 0.05$ for DC formulations, $p < 0.05$ for D formulations) as the capacity for the entrapment of the peptide per liposome is limited and hence remains constant. Consequently, as the same peptide amount was encapsulated in the liposomes at higher initial peptide concentrations, the value for the EE decreased.

As a result of these experiments, formulation DC1b containing cholesterol, pH 9 and a total lipid amount of 28 μmol/ml was chosen for further freeze-drying tests due to the achievement of more homogenous liposomes (PDI = 0.20 ± 0.01, $p < 0.05$) with a higher EE at a lower required DOTAP amount compared with D1 (PDI = 0.31 ± 0.01) (Table 3). This formulation yielded stable and reproducible liposome dispersions with a homogenous size and PDI. The zeta-potential of +56 ± 5 mV which was caused by the DOTAP lipid contributed to its stability.

3.2. Liposome morphology before freeze-drying

The liposome morphology was analysed by transmission microscope (TEM). Three different DOTAP liposome dispersions were investigated and single vesicles were selected for TEM analysis. Next to these smaller liposomes also larger existed which are not shown in the figures.

Fig. 1 shows images of single DC liposomes (Fig. 1A, DC1b without peptide; Fig. 1B, DC3b formulated with 0.9% NaCl solution instead of milli-Q water for the aqueous peptide phase; Fig. 1C, DC3b manufactured with milli-Q water).

The observed liposomes were not completely spherical and had sizes around 100 nm. The multilamellar nature could clearly been observed through all images. In Fig. 1C, their hollow character became apparent because of the bright aqueous core which is surrounded by folded multilamellar membrane structures.

In summary, all results were quite similar and the different formulation additives (active pharmaceutical ingredient or ionic strength) showed no effect on liposome's multilamellar morphology.

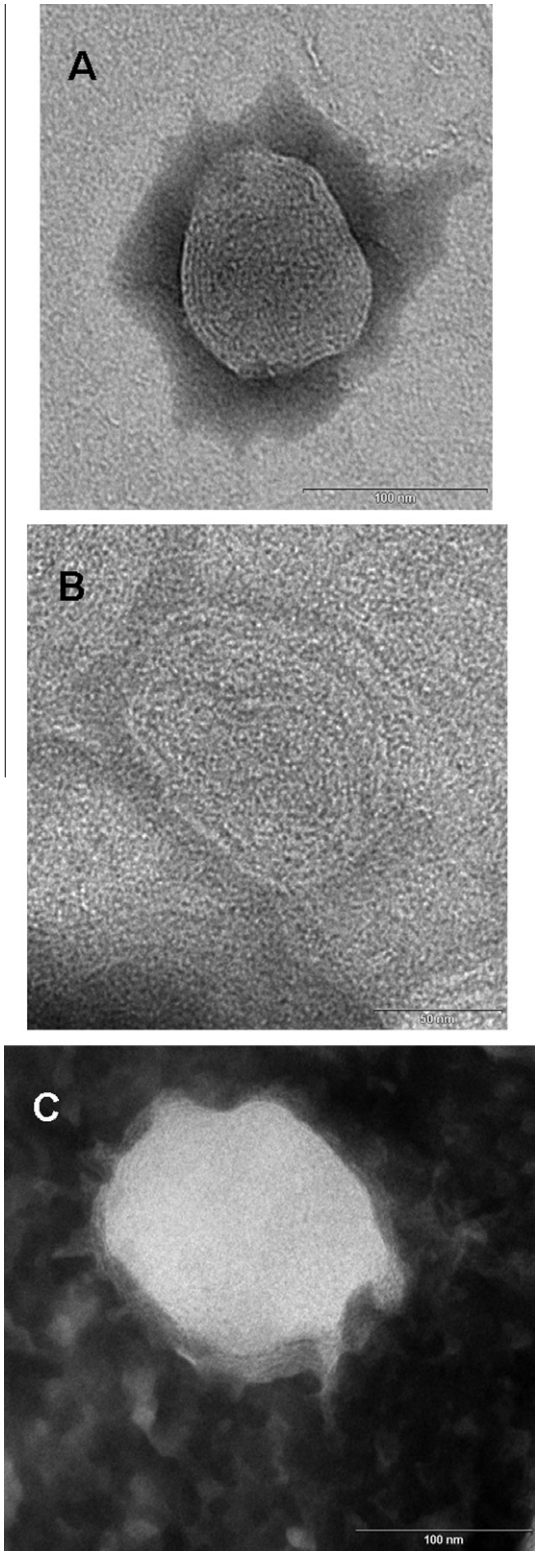

Fig. 1. Images of single DOTAP liposomes taken for morphology analysis by TEM: (A) shows a placebo formulation (DC1b), (B and C) show peptide loaded liposomes (formulation DC3b).

3.3. Effect of different types and amounts of sugar on the collapse temperature of the liposome dispersion

For the development of a freeze-drying process, it is essential to know the maximum allowable product temperature during primary drying. This maximum allowable product temperature is termed collapse temperature T_C and can be determined by special freeze-drying microscopes [10]. The T_C is defined as the temperature during drying when an amorphous product in the frozen state can no longer support its structure. Structural modifications can occur which may be observed by holes or fissures. Consequently, the material can no longer maintain its solidity. Insufficient

stability, high residual water content, poor rehydration time and inhomogeneous cake structures (shrinkage) are resulting from an inadequate primary drying step, which always should run below the T_C of the final product. T_C is always dependent of the entire formulation since all additives and excipients influence the T_C [11].

In this study, the collapse temperature (T_C) of the different DC liposome formulations was determined. Fig. 2 shows the T_C determination of the DC1bS5 liposome dispersion during heating from −40 °C to +20 °C. The corresponding temperatures are given in the image on top of the right side. In Fig. 2A, the black front end demonstrates the sublimation zone. With advanced time and increasing temperature, the front moved down to the inner core of the material (Fig. 2B and C). The dark side of the image corresponded to already dried material; the bright side represented the frozen material. Accordingly, the moving direction was always from the dried to the frozen material. Fig. 2E indicated a beginning structure damage, and in Fig. 2F, broad holes and fissures in the front clearly were observed. Thus, in Fig. 2F, the temperature of −31 °C was above the T_C and the sample underwent collapse. In the order of these events, Fig. 2D showed the last temperature with no observable structure damages and therefore the collapse temperature for DC1b with 5% sucrose (w/v) was determined to be −34.6 °C.

The same procedure was used with trehalose as the cryoprotective agent. Images for T_C determination were equally captured for the DC1bT5 formulation and shown in Fig. 3. The liposome formulation was first frozen to −40 °C and then heated up to 20 °C. The front moved towards the inner core of the sample (Fig. 3A and B). At T = −28 °C (Fig. 3C), the structure underwent collapse and broad fissures appeared. Therefore, for DC1bT5 liposomes, the T_C was −31.4 °C.

Additionally, the influence of the amount of cryoprotective agent was investigated. For this purpose, liposomes formulations containing 3% (w/v) of trehalose (DC1bT3) or sucrose (DC1bS3) were analysed with the Lyostate2 microscope. T_Cs were determined at −31 °C for DC1bT3 and −35.3 °C for DC1bS3. They were similar to those with 5% (w/v) sugar (−31.4 °C for DC1bT5 and −34.6 °C for DC1bS5). Hence, it can be concluded that the time point of collapse is determined by the type of sugar.

In literature, the collapse temperatures for sucrose and trehalose, respectively, were reported to be around −28 °C for trehalose and −31 °C for sucrose, varying on the chosen indications for onset of collapse [11–14]. The T_C for the DC liposome dispersions investigated here trended to lower values, depending on the composition of the formulation. In order to guarantee that the samples maintained the temperature for complete solidification during pri-

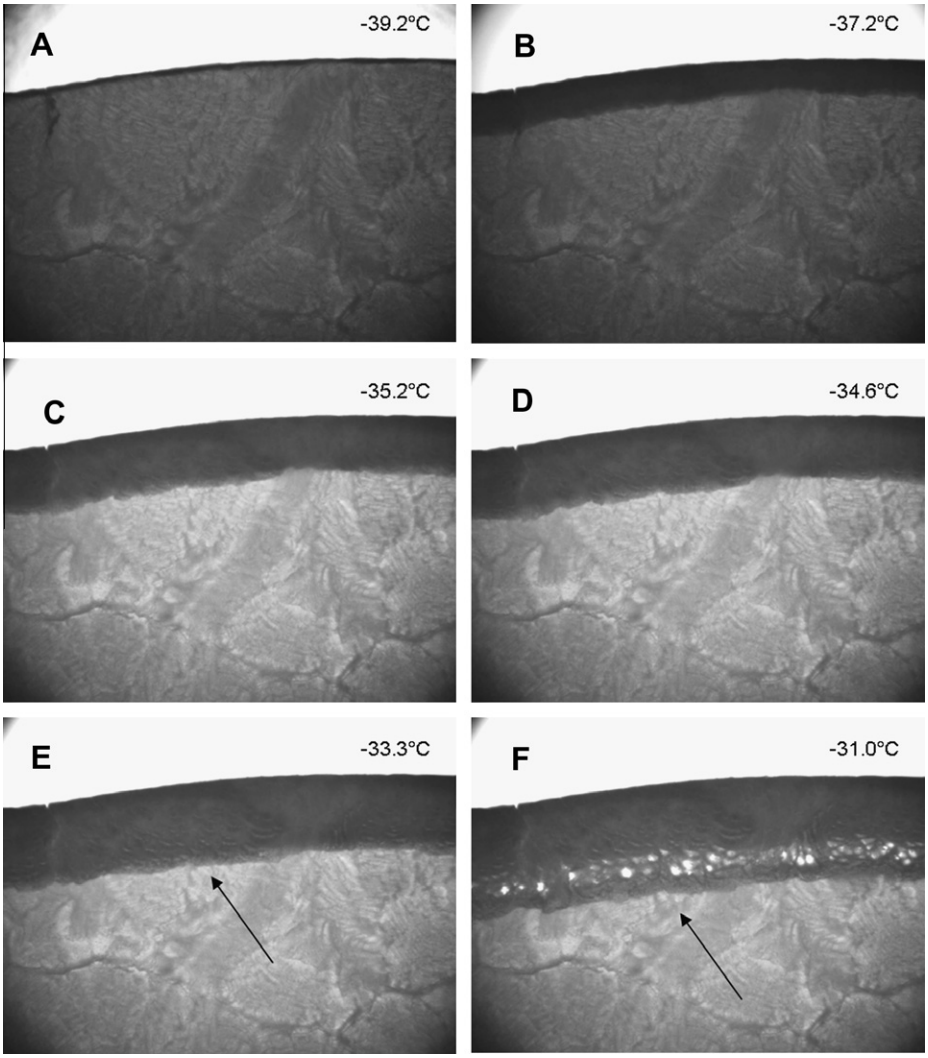

Fig. 2. Images of the moving sublimation front of DC1b liposome formulation with 5% sucrose (w/v) captured with a freeze-drying microscope for T_C determination (magnification 10×).

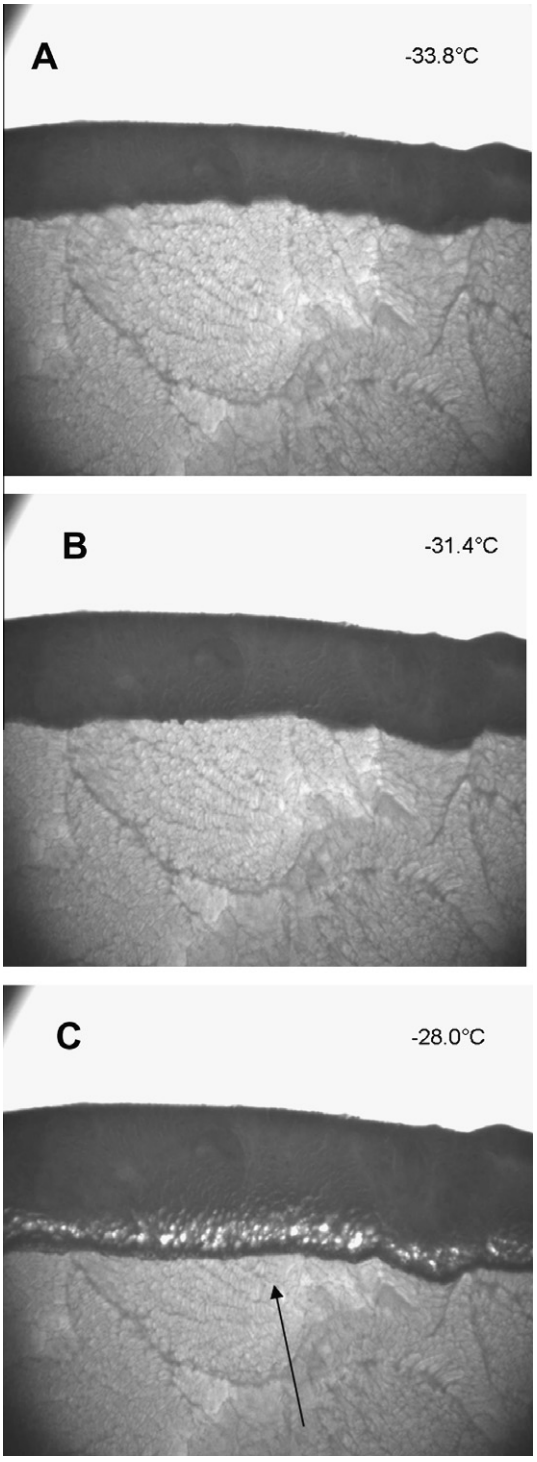

Fig. 3. Determination of T_C for DC1b with 5% trehalose (w/v) analysed with a freeze-drying microscope (magnification 10×).

mary drying, a safety factor of −3 °C was added in further investigations. Consequently, primary drying should be run at a temperature of −38 °C in freeze-drying studies for both cryoprotective agents.

3.4. Effect of freeze-drying

In the first experiment, the liposome formulation DC1bT5 was freeze-dried using three different processes (1–3, shown in Table 2), and the final products (DC1bT51, DC1bT52, DC1bT53; the last number symbolises the implemented process) were compared with each other as well as to the respective properties before the freeze-drying. Experiments were carried out with 3% and 5% (w/v) trehalose, but only the results with 5% trehalose were presented, as no significant difference between these two amounts was observed. Furthermore, 5% trehalose (w/v) is recommended by many scientists [15–17] as suitable cryoprotective agent. For the first experiment, the cryoprotector was added after liposome preparation.

After lyophilisation, the freeze-dried DOTAP-trehalose cakes were examined visually. A total collapse was not observed for any of the three processes. When process 1 was employed, an insignificant shrinkage could be seen at the outer edge of the cake. All cakes could be easily reconstituted with 1 ml of milliQ-water within 10 s, and no visual impurities or aggregates were afterwards observed in the dispersion.

The visual inspection suggested that a successful liposome lyophilisation took place at least after using processes 2 and 3.

To evaluate the physical and chemical properties of the liposomes, the size, PDI, zeta-potential as well as the encapsulation efficiency were determined.

The liposome size significantly decreased after freeze-drying (Fig. 4, $p < 0.05$) in all processes by about 150 nm. This effect seemed to be independent of the respective freeze-drying parameters. The PDI (Fig. 4 right) increased significant for all three cases after lyophilisation (values around 0.4, $p < 0.05$) and also appeared to be not impacted by the different process parameters. Zeta-potential measurements were higher before freeze-drying (around +80 mV; +60 ± 5 mV for process 1) than after freeze-drying (in the range of +70 mV). Generally, the zeta-potential of all lyophilised products leads to stable positively loaded formulations with a high repulsion strength.

To determine the effective amount of entrapped peptide after lyophilisation, the DOTAP liposome dispersions were reconstituted

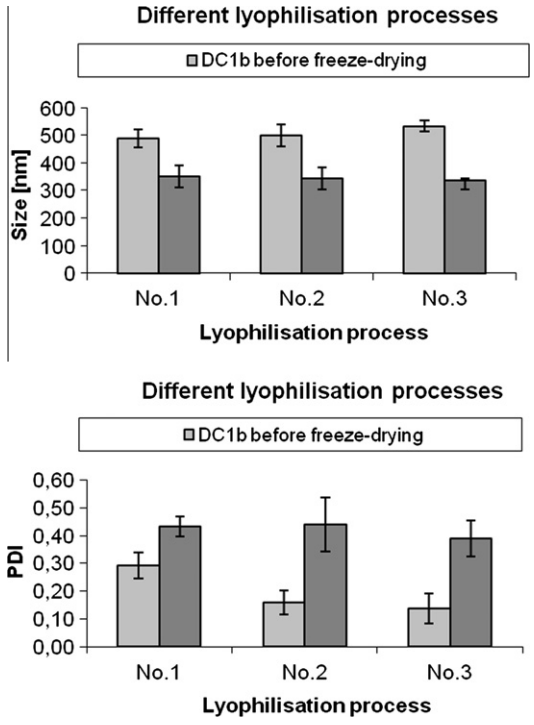

Fig. 4. Size and PDI determinations of DC1b formulation before and after freeze-drying with 5% cryoprotective agent trehalose (DC1bT5) produced by lyophilisation processes 1–3 (Table 2; mean ± SD; $n = 3$).

and separated from leaked peptide by dialyses for 3 h with milliQ-water. The encapsulation efficiency before freeze-drying was ranged between 12% and 30% for all formulations. However, after lyophilisation and additional dialysis, no more peptide was entrapped in the liposomes independently of the process. Therefore, a reorganisation of the lipid structure and accordingly of the whole liposomes obviously has appeared after lyophilisation and reconstitution. Because of these physical changes, the entrapped hydrophilic decapeptide no longer was entrapped in the liposomes inner core and leaked out during dialysis.

This demonstrates that in contrast to literature reports [2,18,19] neither primary drying temperature nor freezing rate had a positive effect on the entrapment efficiency after lyophilisation if the cryoprotector was added after liposome preparation.

As these experiments did not lead to the anticipated results for a steady encapsulated liposome formulation, the preparation procedure was changed by adding the cryoprotector during the liposome preparation, as proposed by Crowe et al., who demonstrated enhanced liposome stability after freeze-drying if the cryoprotective agent resides inside and outside of the lipid bilayers [15,20]. Additionally, the concentration was increased to 7% (w/v) sucrose (DC1bS7) and added to the aqueous peptide solution for re-hydration of the dry lipid film. Higher sugar amounts are recommended by several scientists, because of higher stabilisation effects and improbable drug leakage [19,20]. Sucrose was selected because it is substantially less expensive than trehalose while no significant differences in the PDI were observable ($p > 0.05$). The freeze-dried cakes were reconstituted with milliQ-water and the dispersions were dialysed 3 h with 5 l of 7% (w/v) sucrose solution in order to maintain equal osmotic conditions between liposome core and outer phase during dialysis.

Additionally, the dialysis step before freeze-drying was eliminated in one experiment (Table 4, DC1bS73 with only one dialysis after freeze-drying). Consequently, this preparation still contained free peptide in the outer aqueous phase, which was introduced in the freeze-drying process. This formulation was then compared with the procedure involving two dialysing steps against a 7% (w/v) sucrose solution (Table 4, DC1bS73 with two dialysis before and after freeze-drying).

However, no considerable differences were observed before and after freeze-drying concerning visual appearance, size, PDI and zeta-potential results. Again, the liposomes significantly became smaller in size ($p < 0.05$) with slightly higher PDIs after freeze-drying ($p > 0.05$). The zeta-potential remained constant before and after lyophilisation ($p > 0.05$) and maintaining the high positive surface charge of all formulations. Presumably, independent if the cryoprotective agent was involved in the liposome preparation or added afterwards.

To verify that the freeze-drying process was responsible for the liposome shrinkage, freshly prepared liposomes containing 7% (w/v) sucrose were frozen at −21 °C for 24 h. After thawing, the size, PDI and zeta-potential were analysed and compared with the freeze-dried preparations. The results confirmed the assumption that the freezing process influenced the liposome size [18] as by just freezing without drying the size decreased in a comparable manner from 335 ± 22 nm to 241 ± 15 nm ($p < 0.05$), whereas the PDI (0.34 ± 0.05) and zeta-potential (+57 ± 1 mV) remained constant ($p > 0.05$).

DOTAP liposomes prepared in the presence of sucrose (Table 4, both DC1bS73 formulations "before freeze-drying" with sizes about 330 nm) already before lyophilisation were significantly smaller than those manufactured without sucrose (Fig. 4, DC1b before freeze-drying with sizes around 500–530 nm). Additionally, the liposome homogeneity decreased in the presence of the sugar ($p < 0.05$). Consequently, it can be seen that, the sugar by itself influenced the DOTAP liposome size and PDI.

The pH value (Table 4) remained constant before and after lyophilisation ($p > 0.05$ for DC1bS73 with 2 dialysis steps). It is higher in the DC1bS73 formulation without dialysis before freeze-drying ($p < 0.05$). The viscosity slightly increased after freeze-drying to the same extent in both formulations (Table 4, $p < 0.05$). In addition, the liposome dispersions showed Newtonian behaviour before as well as after freeze-drying. The osmotic conditions were kept constant for all formulations before and after freeze-drying as well as after dialysis (~215 mosmol). The residual moisture content after freeze-drying was determined for both formulations with values <1% (Table 4). Accordingly, with freeze-drying process, 3 dry liposome powders with minimal water content were obtained.

Importantly, the addition of the cryoprotector (sucrose) during liposome preparation enabled the containment of the peptide during the freeze-drying (Fig. 5). In both cases, one or two dialysis steps, the peptide still was entrapped within the liposomes. The

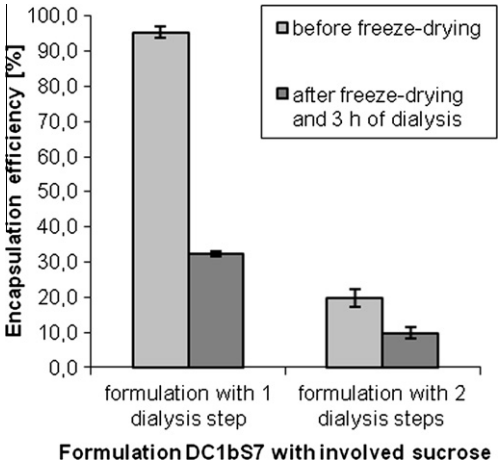

Fig. 5. Encapsulation efficiency results for DC1bS7 formulation with sucrose before and after freeze-drying with process 3 and dialysis (mean ± SD; $n = 3$).

Table 4
Experimental results of freeze-dried DC1bS7 liposome formulation with involved sucrose before and after lyophilisation manufactured with process 3 (mean ± SD; $n = 3$).

Process	DC1bS73		DC1bS73	
	Prepared with 1 dialysis step: only after freeze-drying and containing sucrose		Prepared with 2 dialysis steps: before and after freeze-drying and containing sucrose	
	Before freeze-drying	After freeze-drying	Before freeze-drying	After freeze-drying
Size (nm)	335 ± 22	269 ± 23	326 ± 10	260 ± 13
PDI	0.35 ± 0.06	0.38 ± 0.06	0.27 ± 0.02	0.33 ± 0.04
Zeta-potential (mV)	+57 ± 2	+57 ± 0	+56 ± 0	+56 ± 3
pH	7.8 ± 0.2	6.9 ± 0.1	6.2 ± 0.0	6.3 ± 0.2
Viscosity (mPa s) at 20 °C	2.0 ± 0.0	2.6 ± 0.0	1.8 ± 0.1	2.7 ± 0.2
Residual moister content (%)		0.8 ± 0.2		0.6 ± 0.4

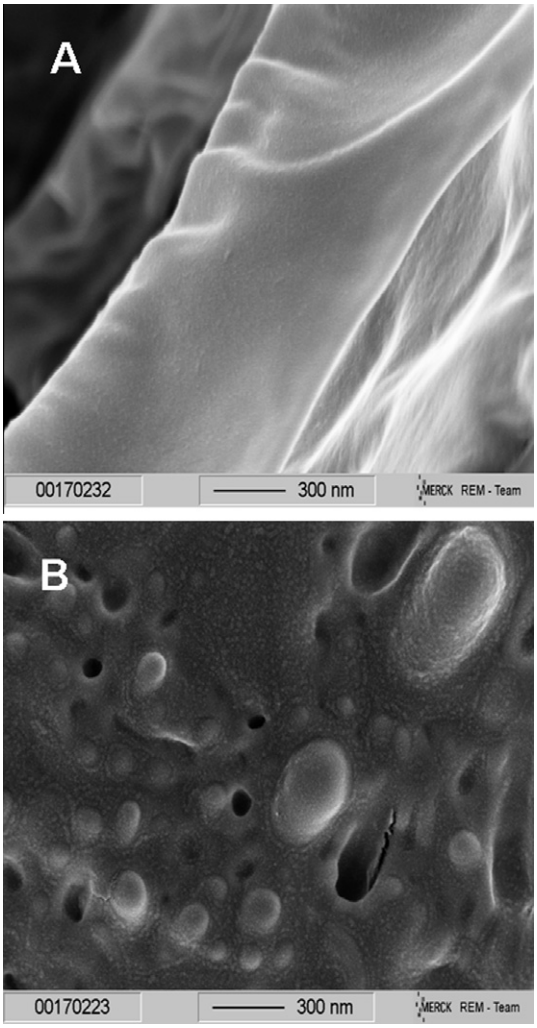

Fig. 6. Images of lyophilised liposomes captured with SEM. Fig. 6A shows the pure freeze-dried sucrose solution; 6B dried lyophilised liposomes prepared with 7% (w/v) sucrose as cryoprotective agent.

sampling point after 3 h of dialysis was chosen to determine the amount of leaked peptide.

When two dialysis steps were carried out (Fig. 5), the sugar did not influence the encapsulation efficiency of the hydrophilic peptide before freeze-drying (EE = 20 ± 3%, $p > 0.05$). However, it still was reduced by 50% down to an entrapment efficiency of 10 ± 2% after lyophilisation ($p < 0.05$) due to the rearrangement of the lipid bilayers during reconstitution of the liposome dispersion after freeze-drying. This reduction in EE could be improved (EE = 32 ± 1%) if the dialysis step before freeze-drying was skipped so that peptide was present within the liposomes and in the outer aqueous phase.

3.5. Liposome's morphology after freeze-drying

To confirm the existence of intact liposomes after freeze-drying, the liposomes were analysed by SEM (Fig. 6). Fig. 6A shows the morphology of a freeze-dried 7% (w/v) sucrose solution without liposomes. A smooth surface without any irregularities or holes was visualised. On the contrary, Fig. 6B shows a micrograph of a dry lyophilised liposome dispersion in the presence of sucrose (DC1bS73, Table 4). In this picture, individual spherical asperities and holes can be seen which demonstrate intact liposomes. The liposomes were almost spherical with diversity in size ranging from 75 nm to 300 nm. This size heterogeneity was also confirmed by PDI measurements performed with a Malvern Zetasizer (Table 4, *z*-average around 260 nm, PDI = 0.33 ± 0.04). The holes next to the liposomes are artefacts caused by sample preparation. With these SEM pictures, the evidence of intact liposomes after freeze-drying was confirmed.

4. Conclusions

This study emphasises the importance of the cryoprotector. Whereas the type of cryoprotector and to some extent even its concentration appear to have none or only minute effects, the time of addition, i.e., prior or after liposome preparation, as well as the presence inside as well as outside the liposomes importantly determines the entrapment efficacy of peptides, in this case specifically for the model decapeptide. Multilamellar DOTAP-cholesterol liposomes were manufactured. In the presence of the cryoprotector sucrose inside and outside of the cationic liposomes, drug leakage could be reduced. The presence of sucrose retained the liposomal character after freeze-drying as evidenced by SEM imaging.

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